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STUDIES ON ERYTHROCYTE MEMBRANE FROM DYSTROPHIC ANIMALS

by

SANJAY W. PIMPLIKAR

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

There is increasing evidence that in muscular dystrophy there may be a basic underlying defect in cell membrane and this may also occur in tissues other than muscle. Erythrocyte membrane has been extensively studied to critically test this hypothesis of generalised membrane defect. In the present work, erythrocyte membrane proteins from dystrophic mice and hamsters have been analysed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two dimensional gel electrophoresis involving isoelectric focusing in first dimension followed by SDS-PAGE. Since the membrane proteins have been implicated in the maintenance of shape, scanning electron microscopy has been performed on erythrocytes to see if alterations in the protein profile are paralleled by alterations in the erythrocyte shape.

On SDS-PAGE, erythrocytes obtained from both dystrophic mice and hamsters have shown the presence of a more intensely stained 55,000 dalton peptide band as compared to the respective controls, suggesting this peptide is present in increased amount in dystrophic animals. Other minor changes have been observed in the protein profile but only this observation has been most consistent. Periodic acid-Schiff staining has indicated that this peptide does not seem to be a glycoprotein and no detectable difference has been observed in the glycoprotein profile of erythrocytes from both dystrophic mice and hamsters. Erythrocyte ghosts membrane proteins have also been analysed using isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension. On such electrophoretograms, dystrophic hamsters have shown the presence of an extra protein spot of 20,000 daltons with an isoelectric point of 4.5. Similar studies of mice erythrocytes are under progress.

The scanning electron microscopy of erythrocytes has shown an increased number of distorted cells from dystrophic animals. Dystrophic mice have shown an increased number of cup shaped "stomatocytes" while dystrophic hamsters have revealed an increased number of star shaped "echinocytes". In control animals these cells have been found to be present in small proportion(less than 10%). These results suggest that in animal muscular

dystrophy cell membrane of non muscular tissues can also undergo marked structural alterations.

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1. INTRODUCTION

The term "Muscular Dystrophy" (MD), which was first used by Erb, refers to a group of neuromuscular disorders which are inheritable and are characterised by a progressive weakness due to degeneration and disappearance of muscle cells (Erb, 1891). However, a case report by Dr. Edward Meryon, which was published almost forty years before the term muscular dystrophy was coined, has been credited as the first accurate and detailed description of MD (Meryon, 1852; Accordo, 1981). Later in 1868, Duchenne published data on 13 cases of *paralysie musculaire pseudohypertrophique* (Duchenne, 1868). In this report, he reviewed the clinical course of the disease, its histology and he described a novel technique to confirm the diagnosis by muscle biopsy, prior to autopsy. Among the several forms of MD, the Duchenne type stands out as a consistent and well characterised disease : X-linked inheritance; onset of symptoms before age three years; legs affected before arms; abnormally high serum enzyme activity; loss of walking ability by early adolescence and death by the third decade.

Despite a long history and intense investigations, the primary cause of MD is not known. Two of the best known hypotheses concerning the aetiology of MD are those which suggest that the myopathic symptoms in MD arise from abnormality of the local circulation (the vascular hypothesis, Demos, 1961; Engel, 1975) or of the motoneurones (the "sick" motoneurone hypothesis, McComas, 1977).

The "vascular hypothesis" suggests that MD is caused by areas of chronic anoxia within a muscle, probably due to capillary occulsion. Thus, a muscle can be damaged or its regeneration impaired by a defect of the microcirculation (Engel, 1975). The "sick" motoneurone hypothesis (McComas, 1977) is based on the assumption that the normal state of muscle fibres is maintained, at least in part, by trophic factors that emanate from the motoneurone terminals. This hypothesis suggests that an abnormality of the motoneurones causes a chronic impairment in the trophic action, resulting in pathological changes in muscle. While both these hypotheses are supported by clinical observations, the

difficulties inherent in carrying out experimental studies on human tissues have made it impossible to test them critically in man.

Another hypothesis, concerning aetiology, of MD is that abnormal muscle cell surface membrane is the primary cause of the disorder (Rowland, 1980). The membrane hypothesis may be stated as follows: the functional genetic fault of MD affects a membrane protein, which is decreased in amount or rendered functionally abnormal because of an altered amino acid sequence. In either case, it results in abnormal composition and altered function of muscle cell surface membrane. Whatever its nature, the membrane abnormality in turn causes the symptomatic weakness and progressive degeneration of muscle cells. (It has been suggested that an abnormal influx of calcium through muscle cell membrane might be primarily responsible for the symptoms of MD; Duncan, 1978; Horrobin *et al*, 1977). However, none of these three hypotheses has been unambiguously proven or disproven and therefore none of them has been dismissed.

The primary reason for the inability to obtain unambiguous support for any of the above hypoyheses is the scarcity of biopsy material from human patients. This paucity of the material imposes limitations on the experimental manipulations either *in situ* or *in vitro* and therefore, many workers have turned their attention to study animal myopathies (Atkinson *et al*, 1980; Cosmos and Butler, 1980; Harris and Slater, 1980). Although there has been much debate about the relevance of such studies, it is generally agreed that a study of those features of the animal diseases that are shared with MD in man will prove to be helpful in understanding more about this disease in man (Harris and Slater, 1980).

The membrane hypothesis is also supported by the observations that enzymes, such as aldolase (Tzvetanova, 1971) and lactate dehydrogenase (Emery, 1964) show increased serum activity in MD patients. These enzymes seem to arise from muscle (Rowland, 1980). It has been proposed that these enzymes escape from cells either by physical interruption of the muscle membrane or that a functional abnormality of unknown nature has rendered the membrane

abnormally permeable, thus resulting in the release of the enzymes (Rowland, 1980).

Biological membranes are mainly composed of lipids and proteins. Of the various models of the biological membranes the one that is most widely accepted is the fluid-mosaic model (Singer and Nicolson, 1972). This model proposes that most of the phospholipids present in the membrane form a bilayer, with the hydrophilic heads of individual phospholipid molecules oriented toward the interior of the bilayer. The "peripheral" proteins remain associated with the bilayer superficially while the "integral" proteins penetrate the bilayer to a varying degree. Since the lipids are assumed to be present in a "fluid state", the membrane components, both the lipid and protein, are capable of movements, as supported by the experimental evidence (Kornberg and McConnell, 1971a; Kornberg and McConnell, 1971b; Frye and Edidin, 1970).

The membrane studies in MD are full of conflicting reports (Rowland, 1980). Of the important factors that might be responsible for these conflicting reports, the most prominent one is the difficulty in isolating and purifying these membranes for their *in vitro* characterisation. It is not yet possible,in any species to purify plasma membrane from basel lamina or collagen, commonly associated with muscle cell surface. Moreover, in MD the muscle is infiltrated by fat and connective tissue and these contaminants may also be present in "sarcolemmal" preparations and distort analysis of what is presumed to be purified muscle cell surface (Takagi *et al*, 1973). These factors, together with the paucity of clinical samples (only about 150 micrograms of membrane protein is obtained from one gram of normal muscle, and biopsies from children with MD rarely provide more than one gram of tissue) turned many investigators to other cells. Fibroblasts have received some attention (Roses *et al*, 1980) but erythrocyte have been investigated more extensively because they can be obtained in larger quantity and it is easy to get pure membrane preparations.

However, this approach has its limitations. Of the 27 reported abnormalities in erythrocytes, there is controversy about all but one of the 18 functions that have been studied in more than one laboratory (reviewed in

Rowland 1980). The irreproducibility of observations is probably due to differences in experimental manipulations such as use of anticoagulants, buffers, freezing and thawing, storage conditions and other as yet, unknown aspects. Moreover, some workers consider this approach to be mistaken because the erythrocyte is a highly specialised cell, that is not only different in structure from muscle cells but is also unable to perform many normal biochemical functions e.g. protein synthesis.

Nevertheless, it is believed that erythrocytes can provide a useful model tissue to study metabolic abnormalities in genetic diseases, involving muscle (Layzer and Rasmussen, 1974). Nagano (1980) has compared membrane proteins from erythrocyte and muscle cells. Band 5 protein of erythrocyte is generally considered as erythrocyte actin and spectrin may be eqivalent to myosin of the muscle. A protein similar to "connectin" in the muscle seems to exist on the inner surface of erythrocyte membrane. So Nagano concluded "...there may be no wonder in thinking that erythrocytes are muscle in disguise". Although biochemical defects in MD are largely unknown, a body of evidence has accrued that describes subtle abnormalities of the erythrocytes in MD (reviewed in *Muscle and Nerve* 3, 1980). This together with the evidence of morphological defects in muscle membrane (Mokri and Engel, 1975) led to a generalised membrane hypothesis that has been recently summarised (Rowland, 1980; Atkinson *et al*, 1980). Thus despite all shortcomings, erythrocyte membrane has been proven to be an extremely useful model.

Several laboratories have reported a variety of abnormalities in erythrocytes from dystrophic patients and animals e.g. abnormal shape (Morse and Howland, 1973; Matheson and Howland, 1974; Miller *et al*, 1976), altered activities of membrane-bound enzymes such as Na-K-ATPase (Mishra *et al*, 1980), Ca-Mg-ATPase (Dunn *et al*, 1981) and adenylate cyclase (Wacoltz *et al*, 1979), abnormalities in membrane protein phosphorylation (Roses *et al*, 1975; Tsung and Palek, 1980), abnormal phospholipid composition of erythrocyte membrane (Kunze *et al*, 1973; Kalafoulis *et al*, 1977), altered osmotic fragility (Kim *et al*, 1980), altered deformability of erythrocyte membrane (Missirlis *et al*,

1981) and altered erythrocyte membrane fluidity (Butterfield, 1981). Interestingly, there are almost an equal number of reports cited in the literature that contradict the above observations (Rowland, 1980).

MD being an inheritable disease, efforts have been made to find mutant proteins. As with the other above mentioned observations, polypeptide profiles of membrane proteins on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) has yielded conflicting results. No significant differences have been observed between the polypeptide profiles of erythrocytes obtained from normal controls and dystrophic patients by Roses *et al*, (1975) and Tsuchiya *et al*, (1981). On the other hand, Das *et al*, (1976) have observed different protein patterns of erythrocyte ghosts from patients with Duchenne MD (DMD). Studies on myotonic goat have shown that a protein of molecular weight (MW) 140,000 was markedly reduced in erythrocyte membranes from diseased animals (Atkinson *et al*, 1980). Very recently Rosenmann *et al*, (1982) have reported a "missing protein" of MW 56,000 and isoelectric point of approximately 6.7, in cultured skin fibroblasts from DMD patients. Burghes *et al*, (1981) have also studied cultured skin fibroblast from patients with DMD and observed differences in the region of MW 35,000-45,000 which they, however, attributed to individual variation rather than to the disease state itself. They have also suggested that earlier reports of similar polypeptide profiles might be due to the low sensitivity of the gel system which might not have detected minor peptide bands. Therefore, in the present investigation, erythrocyte membrane proteins have been studied on SDS-PAGE using a recently developed silver staining technique which is 100 time more sensitive than the conventional coomassie blue staining (Switzer *et al*, 1979). Moreover, high resolution two-dimensional electrophoresis, involving isoelectric focusing in the first dimension and SDS-PAGE in the second dimension has also been performed on erythrocyte membrane proteins. Although this technique has been widely used to study proteins from other systems (Celis and Bravo, 1981), it has not so far been applied to the study of erythrocyte membrane proteins in MD.

It has been postulated that a relationship exists between structure and interaction of the membrane protein and the shape of the membrane (Lux, 1979). Thus, if there is a mutant protein present in erythrocyte membrane, it is likely that this alteration might be reflected as an altered shape of erythrocyte. Therefore, scanning electron microscopic studies have been performed on erythrocyte from normal and dystrophic animals. Although alterations in erythrocyte shape from DMD patients (Miller *et al*, 1976) and from dystrophic mice (Morse and Howland, 1973) have been reported, similar data on hamsters are not available.

In the present study, it has been seen that the protein profile of erythrocytes from both dystrophic mice and hamsters is altered on SDS-PAGE, two-dimensional electrophoresis has revealed an extra protein spot from dystrophic hamsters and SEM studies have demonstrated a marked increase in the number of distorted erythrocytes in both dystrophic mice and hamsters.

2. MATERIALS AND METHODS

2.1 Experimental animal models

2.1.1 Mouse

Breeder mice of strain C57 BL/6J-dy2j (dy2j / +)were purchased from the Jackson Laboratories, Bar Harbor, Maine and were reared in the Biosciences Animal Services, University of Alberta, Edmonton. Homozygous dystrophics (dy2j / dy2j) were recognised by the symptoms (walking inability, muscle weakness) after 15 days of age. The normal mice were either heterozygotes (dy2j / +) or homozygotes (+ / +).

2.1.1.1 Blood collection

Female dystrophic animals and age and sex matched controls, were used in the experiments. About 10-12 month old animals were given ether anaesthesia. Thoracic cavity was opened by an incision and the blood collected by direct cardiac puncture into a heparinised syringe and immediately poured into heparinised vials kept on ice. Usually blood was pooled from four animals in each group.

2.1.2 Hamster

Dystrophic hamsters of strain BIO 14.6 were provided by Dr. J. Elbrink, Dept. of Pharmacology, University of Alberta, Edmonton. This homozygous line of BIO 14.6 was originally derived from BIO 1.50 . Animals of strain BIO 14.6 show various degrees of cardiomyopathy. The cardiomyopathic animals were recognised after 55 days of age by the presence of whitish dots of 1-5 mm diameter located at the lower and lateral surfaces of tongue. Histologically, these lesions were shown to be calcified focal areas of myolysis (Homburger, 1979). Animals of strain BIO 1.50 served as controls

2.1.2.1 Blood collection

Female dystrophic animals of 10-12 months age and corresponding control animals were anaesthesised by giving halothane through nitrous oxide and oxygen (4:1 v/v) flow medium. The animals were then injected with 6% nembutal (150 microliters per 100 gm body weight) and the blood was collected in a heparinised syringe by direct cardiac puncture and poured into heparinised vials kept on ice. Most of the time the blood samples were not pooled and were treated separately.

2.2 Preparation of erythrocyte ghosts

Erythrocyte ghosts from both mouse and hamster were prepared by the method of Fairbanks et al, (1971) which followed the principle of hypotonic lysis defined by Dodge et al, (1963). Freshly drawn blood (usually 1.5 ml from mice and 3 ml from hamsters) was transfered to glass centrifuge tubes kept on ice. All subsequent operations were carried out at 2-4 C. The blood was then diluted with an equal volume of cold 5mM sodium phosphate buffer (pH 8) and 0.15M NaCl (5P,8-S). The suspension was centrifuged in a clinical centrifuge and the supernatant and "buffy coat" were removed. The packed cells were then washed three times by suspension in 5P,8-S and centrifugation as above. After each spin, the surface was thoroughly aspirated to remove contaminating cells. Erythrocytes were then lysed by rapidly adding 10 volumes of cold 5mM sodium phosphate buffer, pH 8 (5P,8). The suspensions were kept on ice for 10 minutes and centrifuged for 15 minutes at 20,000 g (Sorvall centrifuge RC-5B, rotor SS-34 at a setting of 13,000 rpm). The resulting deep red supernatants were aspirated, leaving red translucent pellets of packed ghosts over minute opaque, cream coloured "buttons". The latter were removed at this stage to minimise contamination of the ghosts with proteinases. Each tube was tilted and rotated to allow the ghost pellet to slide off the tightly packed buttons. The ghost pellet was then transfered to another centrifuge tube by using a pasteur pipette. The ghosts were then washed at least three times by suspension in 5P,8 and centrifugation as above, each time

taking care to remove the "buttons". The final pellets were homogenous and white. If not immediately used, the ghosts were frozen-stored at -40 C. The average yield of ghost membrane was about 1 mg protein per 1.5-2 ml of blood. Protein determinations were done by Lowry's method, using bovine serum albumin as protein standard (Lowry et al, 1951).

2.3 Electrophoretic procedures

2.3.1 Sample preparation

To 0.2 ml of ghost suspension (1 to 2 mg protein/ml) enough solid urea was added to bring the concentration to 9 M urea. To this, 1 volume of lysis buffer (9.5 M urea, 2% (w/v) NP-40, 2% ampholytes, pH range 3 to 10 and 5% 2-mercaptoethanol) was added. Once the samples were in lysis buffer, they were either run immediately or frozen. Samples could be stored frozen for up to 2 months without adverse effects.

2.3.2 Isoelectric focussing (First dimension)

Two-dimensional electrophoresis of membrane protein was performed according to O'Farrel (1975) with few minor modifications. Isoelectric focusing (IEF) gels were made in glass tubing (200 mm length, 3 mm inside diameter and 5 mm outside diameter) sealed at the bottom with parafilm. To make 10 ml of gel mixture, 5.5 g of urea were added to a side arm flask, then 1.6 ml of 30% acrylamide stock (28.4% (w/v)acrylamide and 1.6% (w/v) bis-acrylamide), 2 ml of 10% (w/v) NP-40, 1.6 ml of water and 0.5 ml ampholytes, pH range 3 to 10. This gave gel of following composition: 9.5 M urea, 5% acrylamide, 2% NP-40 and 2% ampholytes. The flask was swirled until the urea was dissolved, then 7 microliters of TEMED were added and the solution was degassed under vacuum for 1 minute. Immediately after addition of 10 microliters of freshly prepared 10% ammonium persulphate, the solution was loaded into the gel tubes. Care was taken to avoid trapping bubbles by using a syringe with a narrow gauge hypodermic needle. The tubes were filled to approximately 70 mm from

the top. The gel was overlayed with 8 M urea solution and after 1 to 2 hours, this overlay was removed and replaced with 20 microliters of lysis buffer. The parafilm was removed and the ends of tubes were covered with dialysis membrane, held in place by a 3-mm section of latex tubing. The gels were then placed in a standard tube gel electrophoresis chamber (Bio Rad Gel electrophoresis cell, model 155). The tubes were then filled with 0.02 M sodium hydroxide. The lower reservoir was filled with 0.01 M phosphoric acid and the upper reservoir with 0.02 M sodium hydroxide which was extensively degassed to remove carbon dioxide. The gels were then prerun according to the following schedule; a) 200 volts for 15 minutes; b) 300 volts for 30 minutes; c) 400 volts for 30 minutes. Then the power was turned off, the upper reservoir was emptied, lysis buffer and NaOH were removed from the surface of the gels and the samples were loaded using a Hamilton microsyringe. The samples were overloaded with 10 microliters of sample overlay solution (9 M urea, 1% ampholytes, pH range 3 to 10) then with 0.02 M NaOH and the chamber was refilled. After the samples were loaded, the gels were run at 400 volts for 15-20 hours. The product of the voltage and the time (in hours) was always kept greater than 5,000 and less than 10,000 volt hours.

To remove the gels from the tubes, a 20 ml syringe with a long narrow gauge needle was used to force water between the gel and the glass wall. Later the tubes were connected to another syringe via a short piece of Tygon tubing and the gels were slowly forced out by applying pressure on the syringe. The gels were then either (a) equilibrated and immediately loaded on the second dimension gel, or (b) equilibrated and frozen, or (c) loaded on an appropriate second dimension gel without any treatment.

2.3.3 Equilibration

The IEF gel was extruded into 5ml of SDS-sample buffer (10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS and 0.0625M Tris-HCl, pH 6.8) and shaken at room temperature for 60 minutes.

2.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Second dimension)

In the second dimension the discontinuous SDS gel system of Laemmli (1970) was used.

2.3.4.1 Apparatus

Protean dual slab cell (Bio-Rad Laboratories, Richmond, Calif.) was used to run SDS-PAGE. Glass plates used in making the slab gels were 160mm long and 180mm wide. The glass plate sandwich was formed using a 1.5mm thick spacer. When only SDS-PAGE (without prior IEF) was performed, a Teflon comb (25mm teeth) was used, which formed 15 sample wells in which the samples were loaded.

2.3.4.2 Preparation of slab gels

Gels of 10% acrylamide concentration were used in the second dimension. To make 35ml of gel mixture the following were added to a conical flask: 8.75ml of separating buffer (1.5M Tris-HCl, 0.008M EDTA, pH 8.9), 11.5 ml of 30% (w/v) acrylamide (29.2% acrylamide and 0.8% bis-acrylamide), 0.35 ml of 10% (w/v) SDS, 0.035 ml of TEMED and 10.9 ml of water. This gave the gel the following composition: 10% acrylamide, 0.375 M Tris-HCl, pH 8.9, 0.1% SDS. The solution was stirred and degassed for 5-10 minutes. Polymerisation was started by adding 3.5 ml of 0.3% (w/v) ammonium persulfate which was freshly prepared and degassed for 5-10 minutes. Immmediately after adding ammonium persulfate, the gel mixture was poured into the glass sandwich (see 2.3.4.1). During this step care was taken to avoid trapping bubbles, by using a syringe attached with a narrow plastic tube. The other end of the tube was inserted inside the sandwich and the gel mixture was injected into the sandwich through the tube. The glass sandwich was filled to approximately 20mm from the top and the gel was overlayed with approximately 2 ml of distilled water. The gel was allowed to polymerise for approximately 45 minutes, then the water and the residual unpolymerised gel mixture were removed and the gel surface was thoroughly rinsed with water.

The upper buffer chamber was placed over the sandwich. The stacking gel mixture (5% acrylamide, 0.1% SDS and 0.125M Tris-HCl, pH 6.8) was prepared as follows: 2.5 ml of stacking buffer (0.5 M Tris-HCl, 0.008 M EDTA, pH 6.8), 1.7 ml of 30% (w/v) acrylamide, 0.1 ml of 10% (w/v) SDS, 0.01ml TEMED and 2.7 ml water. This solution was stirred and degassed for 5-10 minutes. Immediately after adding 3 ml of 0.3% (w/v) ammonium persulfate, which was freshly prepared and degassed, the gel mixture was poured onto the separating gel surface all the way to the base of the upper buffer chamber trough and overlayed carefully with distilled water. The stacking gel was allowed to polymerise for 30 to 60 minutes, the water was removed and the gel rinsed with distilled water.

2.3.4.3 Loading Isoelecric Focusing gel onto slab gel

A 1% agarose gel (1 g agarose in 100 ml of SDS sample buffer) was used to keep the cylindrical IEF gel in place and to prevent mixing of the protein zones, which migrate out of the cylinder into the slab. The cylindrical gel was placed on a piece of parafilm straightened and placed close to the edge of the parafilm and parallel to the edge. One milliliter of melted agarose solution (80 C) was then poured in the notch above the stacking gel. With the aid of a spatula, the cylindrical gel was quickly transferred from the parafilm into this agarose solution.

2.3.4.4 Electrophoresis in second dimension

About 10 minutes were allowed for the agarose to set and then electrode buffer (0.025M Tris base, 0.192M glycine and 0.1% SDS) was poured into the upper buffer chamber and also into the lower buffer tank. The slab gel together with the upper buffer chamber was then transferred to the lower buffer tank. For efficient cooling, enough electrode buffer was added to the lower buffer tank to immerse the slab gel completely. The gels were run at 20 mA per slab gel (sometimes two slab gels were run simultaneously) until the dye front reached the separating gel. The current was then increased to 30 mA per slab gel until the dye front reached the bottom of the gel. The

running time was about 5 hours.

When IEF gels were directly put on second dimension SDS-PAGE, without prior equilibration in SDS sample buffer, a minor modification in the above procedure was adopted. After securing the IEF gel on the stacking gel with agarose, the upper buffer chamber was filled with "high SDS elctrode buffer" (2% SDS instead of 0.1% SDS) and the lower buffer tank with the regular electrode buffer. The gels were then run at 20 mA/ slab gel for 20 minute. The power was turned off, the high SDS buffer was removed and replaced with the regular running buffer. The gels were then run as usual.

When only SDS-PAGE was performed, i.e. without prior IEF, the samples were prepared as follows: 1 volume of the ghost suspension (1-2 mg protein /ml) was mixed with either 1 volume or 2 volumes of SDS sample buffer and heated at 95 C for 3 minutes. The samples were allowed to come to room temperature and if not loaded on the gel immediately, were frozen stored at -40 C. Sample wells were formed in the stacking gel by inserting a Teflon comb into the stacking gel immediately after the stacking gel mixture was poured on the separating gel (before placing the upper buffer chamber on the glass sandwich). After 30-40 minutes, the comb was removed,the gel surface rinsed with water and the samples were loaded (usually 50-70 microliters containing 40-80 micrograms of protein) in the wells using a Hamilton syringe.

2.3.5 Staining procedures

2.3.5.1 Coomassie blue staining

The coomassie blue staining was performed according to Weber and Osborn (1975). Gels were stained in staining solution (0.25% coomassie brilliant blue R 250, 50% methanol and 10% glacial acetic acid) for 6-8 hours at room temperature. To remove the excess stain, gels were soaked in destaining solution (5% methanol in 10% glacial acetic acid) till the background was clear.

2.3.5.2 Silver staining

The silver staining procedure was performed according to Wray *et al*, (1981). At the end of electrophoresis run, the gel slabs were removed from the electrophoresis cell, the spacers removed and the gel plates were pried apart with a plastic comb. The gels were briefly rinsed in water and then soaked in 50% methanol overnight with at least two changes. The gels were then stained in ammonical silver nitrate reagent for 15 minutes with constant gentle agitation. (The silver nitrate reagent was prepared as follows: 4 ml 20% (w/v) silver nitrate solution was added dropwise into a flask containing 21 ml of 0.36% sodium hydroxide + 1.4 ml of 14.8 M ammonium hydroxide with constant swirling and the volume was made up to 100 ml -this solution must be used within 5 minutes) The gels were washed in deionised water with gentle agitation for 5 minutes. The washing was repeated once more and the gels were developed by soaking in developer (freshly prepared by adding 0.5 ml of 38% formaldehyde to 5 ml of 1% citric acid and making up the volume to 1 liter.) until bands appeared. Usually the bands took less than 10 minutes to reach the desired intensity. The stain development was stopped by washing the gel with water and by placing it in 45% (v/v)methanol+ 10% (v/v)acetic acid.

2.3.5.3 PAS staining

The Periodic Acid-Schiff staining was performed according to Neville and Glossmann, (1974). Gels were fixed in methanol washing solution (Methanol 40%, acetic acid 7%, water 53% all v/v) overnight with gentle agitation. The solution was changed and the washing continued for at least 8 more hours.

The gels were then placed in cold oxidising solution (1% w/v periodic acid in 7% v/v acetic acid) and incubated in the dark at 4 C for . 2 hours. The oxidised gels were then returned to 7% acetic acid wash solution and washed for 1 hour. The solution was changed and the washing continued overnight. A third wash for 1 hour was performed and the gels were transfered to cold Schiff reagent and incubated for 2 hours at 4 C in the dark. The gels were then washed with bisulphite wash solution (1% sodium bisulphite in 0.1N HCl) until the maximum colour was developed.

2.3.6 Measurement of pH gradient

The IEF gel was cut into 1 mm sections and 5 successive sections were pooled together and placed into individual vials containing 1 ml of degassed water. These vials were capped, shaken for 2 to 5 minutes then kept at room temperature overnight. The following day, the pH was measured using a pH meter.

2.3.7 Photography and drying of the gels

Immediately after staining, the gels were photographed, while still wet. Kodak Panchromatic-X (ASA 32) film was used.The photographs were printed on Kodabromide F5 paper.The gels were dried at 90-95 C under vacuum. The negatives were scanned in "Double beam microdensitometer (Joyce, Loebel and Co. Ltd., Gateshead-on-Tyne, U.K.)

2.3.8 Cleaning of glass plates and tubes

The glass plates were rinsed with water then washed with "Alconox" (Alconox Inc., New York, USA), rinsed thoroughly and allowed to dry overnight in an oven. After each 4 to 5 runs, the glass plates were soaked overnight in chromic acid. The glass tubes were rinsed with water, soaked overnight in concentrated nitric acid, rinsed with water followed by 95% ethanol, followed by acetone. Finally the tubes were again rinsed with water and oven-dried overnight.

2.3.9 Chemicals

All the chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. except Ammonium persulfate (from Kodak Eastman, N.Y.), TEMED (from Matheson Coleman and Bell, N.J.) and Sodium dodecyl sulfate (K and K Laboratories, N.Y.). Methanol used in silver staining was obtained from Baker Chemical Co., N.J. .

2.4 Scanning electron microscopy

The samples for SEM were prepared according to Miller *et al* (1976). Blood was drawn by cardiac puncture, through a 23 gauge needle attached to a heparinised disposable syringe. One drop of blood was immediately placed into a polypropylene test tube, containing 10 ml of 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.4. The tube was gently inverted, several times to assure distribution of the cells in a large volume of fixative. After settling for one hour at 4 C, the fixative was pipetted off and the cells were collected from the bottom of the tube in a small drop of glutaraldehyde with a pasteur pipette. The cell suspensions were allowed to settle on collagen-coated coverslips for one hour at room temperature. They were dehydrated through a graded series of ethanol and then air-dried. Centrifugation was avoided in all the steps. The coverslips were mounted on specimen stubs using silver conductive paint (Acme Chemicals and Insulation Co., New Haven, Connecticut) and sputter coated with gold target, approximately 150 Angstrom units thick, in Edwards S 150 B sputter coater. The cells were viewed in a Phillips SEM 505 at 25 kv. The samples were coded and photographs were taken of random fields. The micrographs were recorded on Kodak plus X black and white film, ASA 100 using a 35 mm camera and were printed on Kodabromide F3 paper. The cells were counted by visual inspection.

3. RESULTS

3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

On SDS-PAGE, proteins are separated according to their molecular weights. The theoretical basis of this phenomenon is discussed in appendix I.

Fig. 1 demonstrates a typical electrophoretogram of gel in which proteins from different sources were loaded on the gel under the conditions described in "Materials and Methods". The peptide bands are sharp, flat and stacked one above another. Fig. 2a shows separation of different peptide chains loaded in a single sample well (well D). The identity of each band was established by running each protein in separate sample wells. Fig. 2b shows a similar set of proteins but stained with silver stain instead of coomassie blue. With silver stain bovine serum albumin is not at all stained (arrow). Therefore, in the subsequent runs phosphorylase b is used. The molecular weights for these polypeptide chains are taken from "CRC Handbook of Biochemistry. Selected Data for Molecular Biology".

The dependence of the mobility of polypeptide chains on the logarithm of the molecular weight is illustrated in fig. 3 . Most of the points fall close to the straight line showing that separation in the range of 15,000-70,000 is excellent and that molecular weight of any protein in this range can be determined with a fair amount of accuracy from the electrophoretic mobilty of the unknown protein.

Electrophoresis of ghost membrane protein generally yields a pattern in which six well resolved bands predominate (fig. 4). These bands are arbitrarily labeled 1 to 6 according to the notation of Fairbanks *et al* (1971). Mouse eythrocytes also yielded a peptide profile similar to that shown in fig. 4. Human erythrocytes also yield a pattern of six predominant bands (Fairbanks *et al* 1971), and most of these bands have been well characterised. Bands 1 and 2 (apparent mol. wts. 240,000 and 220,000 daltons, respectively) are refered to as "spectrin". The next major band, band 3, has an apparent mol. wt. of 90,000 -100,000 daltons, and seems to be composed of a number of

heterogeneous molecules (Roses and Appel, 1973). Band 5, or erythrocyte actin, has an apparent mol. wt. of 45,000 and band 6 which comigrates with erythrocyte glyceraldehyde-3-phosphate dehydrogenase has an apparent mol. wt. of 33,000 daltons. Some of the minor bands have also been characterised such as, band 4.1 of apparent mol. wt. 77,000has been implicated as one of the important cytoskeletal proteins (Branton, 1981) and bands of 4.5 region have been implicated in the monosacharide transport (Jones and Nickson, 1981)

3.1.1 Mouse Erythrocyte Ghosts

3.1.1.1 Silver staining

Figure 5 shows results of electrophoresis of ghost membrane protein from normal and dystrophic mice. This pattern of six well resolved bands was reproducible and close observation revealed many more minor bands after staining with silver stain. When the peptide profiles between normal and dystrophic mouse erythrocyte ghosts were compared, a difference in the range of 55,000-60,000 Dalton was observed (arrow). This polypeptide was always found to be more intensely stained in the dystrophic animals compared to that in the controls. This difference could be due to an increase in the quantity of the peptide and is clearly illustrated in densitometric traces (Fig. 6). There were other minor differences found occasionally and they were probably due to individual animal variations. However, increased amount of 55,000 dalton peptide was consistently observed in all five but one specimens studied.

3.1.1.2 PAS Staining

When stained for carbohydrates by PAS procedure, electrophoretograms of erythrocyte ghosts gave the pattern illustrated in figure 7. An intense staining reaction was seen just behind the tracking dye and it was presumably due to glycolipids (Fairbanks et al, 1971). Four PAS positive bands were consistently observed in the region of 85,000, 55,000, 40,000 and 28,000. Fairbanks et al (1971) have reported three PAS positive bands with the apparent molecular weights of 83,500, 45,600 and 25,500 from human erythrocytes.

Four pairs of normal and dystrophic mice were studied and no detectable differences in the PAS positive bands were observed.

3.1.2 Hamster Erythrocyte Ghosts

3.1.2.1 Silver Staining

Figure 8 shows the results of electrophoresis of ghost membrane proteins from hamsters. As with the mouse erythrocyte ghosts, hamster ghosts also gave a well reproducible pattern of six major bands. In the case of hamsters as well, the dystrophic animals showed an enhanced staining of the 55,000 dalton band and this difference was observed in four out of the six specimens studied. The difference however, could not be picked up by the densitometer due to inherent low sensitivity of the densitometer.

3.1.2.2 PAS Staining

PAS staining for carbohydrates showed almost an identical pattern to that of mouse erythrocyte ghosts (fig. 9). Four PAS positive bands were observed in hamster also. Though silver-stained electrophoretograms of hamster erythrocytes showed a difference in the the 55,000 dalton band, no differences were observed in the PAS-stained electrophoretograms from all the three pairs of animals studied.

3.2 Two-dimensional electrophoresis (2-DE)

SDS-PAGE has been proved to be extremely useful analytical technique for the separation and mol. wt. determination of protein species from complex mixtures. However, for extensive analysis of complex systems, 2-DE has proved to be indispensible. Since in 2-DE each dimension can separate proteins according to independent parameters, optimal separation can be achieved. In the present investigation IEF has been used in the first dimension which separates proteins according to charge and SDS-PAGE in the second dimension which separates according to mol. wt. . A brief analysis of the

theoretical basis of charge separation phenomenon is given in appendix II.

For good resolution of polypeptides on IEF gels, it is important that pH gradient in the gel be linear. Fig. 10 shows that the pH gradient in the gels used was fairly linear throughout the length of the gels. Only at the two ends of gels (extreme pH values), the pH linearity was not observed. This phenomenon has been observed by others also (O'Farrell, 1975).

Fig. 11 clearly demonstrates that 2-DE allows the erythocyte membrane to be resolved into a much larger number of components (up to 50)than are revealed by one dimensional electrophoresis (15-20 bands). This has been observed earlier by others as well (Rubin and Milikowski, 1978; Thompson *et al* 1980). The characterisation of many of these components is far from complete at present (Jones and Nickson, 1981).

3.2.1 Mouse Erythrocytes Ghosts

Fig. 12 shows two dimensional electrophoretograms of mouse eythrocyte ghost membrane protein obtained from normal (fig. 12a) and dystrophic (fig. 12b) animals. The dystrophic animals showed increased staining of two peptide spots (shown by arrows) of approximate mol. wt. 30,000 and isoelectric point (pI) 4.2 and approximate mol. wt. 22,000 and pI 7.0 . No detectable difference was found in the 55,000 dalton region. Only one sample has been studied so far and the study is under progress.

3.2.2 Hamster Erythrocytes Ghosts

2-DE of hamster erythrocyte ghosts showed many more spots than that shown by mouse erythrocyte ghosts (fig. 11). Close inspection of electrophoretograms from normal (fig. 13a) and dystrophic erythrocyte ghosts (fig. 13b) revealed only one difference. There was an additional spot present in the region of 20,000 dalton and pI 4.5 in the electrophoretograms obtained from dystrophic ghosts (arrow). This difference was consistently observed in all the four pairs of animals so far studied. No detectable differences in the region of 55,000 daltons were observed.

3.3 Scanning electron microscopy

SEM has been used to examine the surface of specimens at high resolution. In principle it works much like a transmission electron microscope the only difference being that backscattered (rebound) and secondary electrons are used to form the image rather than deflected electrons. A brief description of image formation in SEM and the limitations of this technique is given in appendix III.

Erythrocytes normally appear as biconave discocytes, though in a normal animal a minor percentage of cells can be present in a shape other than the typical biconcave (Miller *et al* 1976). Some of the most commonly observed shapes of erythrocytes are illustrated in fig. 14. A normal biconcave discocyte is seen in fig. 14a while fig. 14b shows a knizocyte (triconcave cell). These cells resemble the discocytes; only difference being that knizocytes have an additional concavity. Fig. 15c shows a typical cup shaped stomatocyte; the term stmatocyte being derived from the mouth like appearence. Fig. 15d shows a spicular echinocyte; their surface bears projections giving them a star like appearence.

3.3.1 Mouse erythrocytes

In controls, the majority of erythrocytes appeared as typical biconcave discocytes (fig. 16a). A few stomatocytes and rarely knizocytes or echinocytes were observed. The sparse abnormal forms among cells from normal animals could be an artifact of fixation produced by altered sensitivity of older cells (Miller *et al* 1976). In comparision, dystrophic animals showed much larger population of stomatocytes (fig 16b). The percentage varied with the individual animals (Table 1), but with the exception of one animal, the number of stomatocytes was always at least five times greater in the dystrophic animals than in the controls. In the normal animals more than 90% cells were biconcave discocyte.

3.3.2 Hamster erythrocytes

. Fig. 17 shows a field of cells obtained from normal (fig. 17a) and dystrophic animals (fig. 17b). As in mice, very few erythrocytes with irregular shapes were observed in normal hamsters. A large proportion of the cells in the dystrophic animals (fig. 17b) showed surface irregularities involving variable protrusions from the cell surface. They resemble echinocyte and have been observed earlier (Morse and Howland, 1973) in dystrophic mice. In the normal animals around 10% of the total cells were distorted while in the dystrophic animals this number was increased to almost 60% while the remaining cells were biconcave discocytes (Table 2). Very few, if any, stomatocytes were observed either in the controls or in the dystrophic animals.

4. DISCUSSION

Based on the data derived from SDS-PAGE analysis, it can be stated that in both mice and hamsters a protein of 55,000 daltons shows an increase in amount in dystropic erythrocyte ghosts. This peptide does not belong to the major 6 bands observed in erythrocyte membrane and the significance of this finding remains unknown.

Earlier reports on the polypeptide composition of erythrocytes in MD have yielded conflicting results. Tsuchiya *et al*, (1981) and Roses *et al*, (1975) found no differences between peptide profiles of normal and dystrophic ghosts from humans. Das *et al* (1976) observed different protein pattern of ghosts from patients with MD. However, details about the differences were not given. Recently Rosenmann *et al*, (1982) reported a "missing protein" in fibroblasts from DMD patients. This protein has a molecular weight of 56,000 daltons and isoelectric point of 6.7, and was found to be consistently absent in dystrophic patients.

The differences in various reports on protein analysis could be due to the differences in the techniques used by different workers. Erythrocyte membrane is susceptible to proteolytic degradation during the experimental manipulations (Fairbanks *et al*, 1971). In the present study, care was taken to minimize the chances of such artifacts by keeping isolation time to a minimum and removing contaminating cells, which exhibit proteolytic activity (e.g. leukocytes), at an early stage of the isolation. The presence of protease inhibitors does not seem to be very effective since these inhibitors are highly specific for their respective proteases (Bhakdi *et al*, 1975) and therefore, use of only one protease inhibitor will not necessarily prevent proteolysis. Another variable of critical importance is the sample preparation. A slight variation in the protocol of sample preparation can give rise to a different peptide profile on the electrophoretogram (Rubin and Milikowski, 1978). Moreover, different staining procedures might show different electrophoretogram profiles for the same protein mixture. Though coomassie blue stains bovine serum albumin, silver stain fails to do so (fig 1a and 1b). During the reaction of silver

cations with protein in an alkaline environment, protein amino groups are complexed with silver (Wray *et al*, 1981). Thus, if fewer number of free amino groups are available in the protein, it will not be stained properly with this procedure of silver stain. Similarly, none of the PAS positive bands observed in human erythrocytes showed up in the gel when stained with coomassie blue stain (Fairbanks *et al*, 1971). Thus, any report of a "different peptide pattern" or a "similar peptide pattern" is subject to all of these variables and the peptide profile need not necessarily reflects the true *in vivo* situation. Nevertheless, from the present investigation it is clear that the erythrocyte membranes obtained from normal and dystrophic animals when subjected to identical experimental manipulations (membrane isolation, sample preparation, gel electrophoresis, etc) exhibited a difference in the protein patterns indicating that these two membrane populations, at least in some respects, are different.

Two-dimensional electrophoresis is capable of resolving a complex protein mixture into a much larger number of components than one-dimensional electrophoresis (O'Farrel, 1975). Results from the present investigation showed that ghosts proteins are indeed resolved into more components on 2-DE. In the case of hamsters, an additional protein spot was observed on the electrophoretograms of dystrophic ghosts. This difference was not observed on one dimensional SDS-PAGE due to the presence of another protein spot with similar mol. wt. (20,000 daltons). Obviously these two peptides of similar mol. wt. have different isoelectric point values. In the preliminary experiments on mice eythrocyte ghosts, some differences were observed on 2-DE electrophoretograms but this observation requires confirmation.

Though SDS-PAGE has been used extensively to study membrane protein profile in MD, 2-DE had not been used until recently. This is probably the first report of two-dimensional electrophoretic studies on erythrocyte membrane in MD. Recently, Rosenmann *et al*, (1982) reported a "missing protein" from fibroblasts of dystrophic patients using 2-DE. Earlier studies on fibroblasts obtained from patients with DMD showed no difference on one-dimensional

SDS-PAGE (Burghes *et al*, 1981). This shows that 2-DE is far more useful than SDS-PAGE in screening for proteins.

Over the past few years, erythrocyte membrane proteins have been analysed by using different two-dimensional gel systems (Falk *et al*,1976; Rubin and Milikowski, 1978; Thompson *et al*,1980). Falk *et al*, (1976) reported resolution of 30 individual components using urea, NP-40 IEF followed by SDS-PAGE. Using the similar system, up to 50 peptide spots have been resolved in the present study (fig. 11). However, from the electrophoretograms it is clear that considerable amount of protein has not entered the IEF gel and has appeared as a smear on 2-D gels. This phenomenon has been observed by others (Bhakdi *et al*,1975) and can be prevented by using SDS during IEF sample preparation. Rubin and Milikowski, (1978) by using SDS during IEF sample preparation resolved over 200 peptides. They suggested that SDS, by removing spectrin and band 3 proteins selectively by aggregation prior to entrance into the gel, allowed minor components to enter the IEF gel. They did not, however, rule out the possibility that some of the spots obtained on the electrophoretograms were in part artifacts. This and the possibility that SDS binding might affect the migration of protein (by altering the native charge) were the reasons for not using SDS in IEF samples in the present study.

Artifactual spots can also be seen on electrophoretograms due to induced charge heterogenity. Isocynate, formed by decomposition of urea, might result in carbamylation of proteins and therefore a loss of free amino group (O'Farrel, 1975). The loss of a free amino group, below pH 8.5, results in a unit change in the charge of a molecule and hence a shift in pI. Thus, deamination artifact results in a uniform series of spots (Anderson and Hickman, 1979). The following precautions were taken to prevent carbamylation in the present work: all urea solutions were stored as frozen aliquots; ampholytes were present in all urea solutions which contact the protein; once the protein was dissolved in urea solution, the time during which it was not frozen was kept to a minimum ; and finally, the IEF gels were prerun to remove isocynate, pKa 3.75 (O'Farrel, 1975). Since a uniform series of spots is largely absent

from the electrophoretograms, it is highly unlikely that the additional spot observed from dystrophic ghosts is a deamination artifact.

The present SEM studies showed that a large number of erythrocytes from both dystrophic mice and hamsters have abnormal shapes. This observation is consistent with the earlier reports of alterations in erythrocyte shapes from dystrophic mice (Morse and Howland, 1973) and patients with DMD and myotonic MD (Miller *et al*, 1976; Matheson and Howland, 1974). Thus, the phenomenon of erythrocyte shape alteration seems to be common in various dystrophies.

Quantitation of cells showed that both in mice and hamsters, the normal animals have at least 90% of biconcave discocytes and few (around 10%) distorted cells. Most of the distorted cells were stomatocytes and very few were knizocytes (triconcave cells). Though all the dystrophic animals examined showed some typical biconcave cells, distorted cells were present in increased numbers. Interestingly, though both dystrophic mice and hamsters showed an increase in the number of distorted cells, the nature of the distortion was different in these two species. Dystrophic mice showed an increase in the number of stomatocytes while dystrophic hamsters showed an increase in the number of echinocytes. There was considerable variation in the percentage of the distorted cells in both dystropic mice and hamsters (46%-73% in hamsters and 35%-67% in mice). Matheson and Howland (1974) also observed large variations in the percentage of the distorted cells in patients with DMD (20%-98%). This shows that both dystrophic patients and dystrophic animals can show large variations in the number of the distorted erythrocytes.

This phenomenon of altered erythrocyte shape has also been observed in many clinical disorders, other than MD. Altered shapes of eythrocytes have been reported in patients with congenital abetalipoproteinemia (Brecher and Bessis, 1972); sickle cell anaemia (Jensen and Lessin, 1970; Rice-Evans and Chapman, 1981); beta-thalassaemia (Rice-Evans and Dunn, 1982); hereditary spherocytosis (Thompson and Maddy, 1981) and many other clinical conditions (Brecher *et al*, 1973). The molecular mechanism of the shape alteration in

most of these conditions is not known.

The possible mechanism of altered erythrocyte shape in MD also remains unknown. It has been speculated that an abnormal phospholipid composition of erythrocyte membrane in MD might be responsible for the altered shape (Matheson and Howland 1974). Since, treatment of normal erythrocytes with calcium at a high pH has been shown to induce similar alterations in shape as found in MD (Weed and Chailley, 1973), it is possible that the changes in membrane lipid produce an abnormal pattern of cation distribution (Howland and Challberg, 1973) which in turn induces shape alterations. Alternatively, changes in membrane lipid profile may bring about the alterations by directly influencing different membrane proteins and their interaction with the cytoskeletal elements (Rice-Evans and Dunn, 1982).

Lux (1979) has suggested that membrane deformability, flexibility and morphology are primarily dependent on and controlled by a submembranous protein scaffolding composed of spectrin, actin and band 4.1. Spectrin band 2 phosphorylation has been shown to be consistently higher in erythrocytes from patients with DMD (Roses and Appel, 1976). Thus, it is possible that altered levels of spectrin band 2 phosphorylation might be ultimately responsible for the altered shape of erythrocyte in MD (Rice-Evans and Dunn, 1982). In hereditary spherocytosis as well, the underlying defect, that induces the shape transformation, has been attributed to abnormalities in the control of spectrin band 2 phosphorylation (Thompson and Maddy, 1981).

The present investigation has indicated that erythrocytes from both dystrophic mice and hamsters, show altered polypeptide profile as well as altered erythrocyte shape. However, the altered proteins from dystrophic animals need to be characterised, both in terms of structure and function, before any link between these two findings could be sought.

Alterations similar to those found in the present investigation can be produced *in vitro* under nonphysiologic conditions. Stomatocytes can be readily formed by exposing normal discocytes to low pH (Bessis and Bricka, 1950), cationic detergent like agents (Deutick, 1968), and chlorpromazine (Weed and

Bessis, 1973). Echinocytes can be produced by many factors, among them are elevated pH (Ponder, 1948), anionic detergent like agents and exposure to lysolecithin (Deuticke, 1968), intracellular ATP depletion (Weed *et al*, 1969) and cell ageing (Brecher and Bessis, 1972). Thus, it seems that erythrocytes can react to seemingly unrelated perturbants with a limited number of shape transformations.

The easy susceptibility of erythrocytes to undergo shape transformation raises the possibility that these erythrocytes from dystrophic animals might have normal biconcave shape *in vivo* and they undergo shape transformation much more readily than the erythrocytes from normal animals. The mean life span of erythrocytes in dystrophic hamsters has been shown to be slightly longer (57.3+0.7 days) than in the controls (50.1+1.6 days, Toffelmire and Boegman, 1980). This suggests that at any given time dystrophic hamsters will be having more aged cells than their normal counterparts. Thus, as shown by Brecher and Bessis (1972), cell aging might be in part responsible for the membrane shape distortion observed in the present investigations. Since "age" corrected population of erythrocytes can not be obtained without extensive manipulations, usually involving differential centrifugation, findings from such studies will be difficult to interpret for its *in vivo* relevance. It is suggested that the small proportion of abnormal cells (usually less than 10%) observed in normal individuals may be an artifact of fixation produced by the altered sensitivity of older cells. It should be pointed out, therefore, that the alterations in the shape of erythrocytes seen in dystrophic animals are not necessarily an evidence of their existence as such *in vivo*. This could be the result of intrinsic biochemical membrane differences that respond to fixation differently.

It is interesting to note that in the present studies dystrophic mice have shown increased number of stomatocytes (fig. 16b) while dystrophic hamsters have shown that of echinocytes (fig. 17b). Miller *et al* (1976) have reported an increase in the number of stomatocytes while Matheson and Howland, (1974) have reported an increase in the number of echinocytes from patients with DMD. Miller and coworkers suggested that this discrepancy was probably due

to the differences in the technique of sample preparation (procedure of Miller and coworkers avoids centrifugation). In the present study the protocol given by Miller and coworkers was followed which yielded stomatocytes in dystrophic mice and echinocytes in hamsters. Obviously, the discrepancy seen earlier is due to some factors other than the 'differences in the technique of sample preparation'.

In summary, these findings of altered polypeptide profile of eythrocytes from both dystrophic mice and hamsters together with the findings of increased number of distorted erythrocytes in these dystrophic animals suggest that serious consideration should be given to the hypothesis linking genetic dystrophy and systemic alteration of cellular membrane. A defect of membrane could result in a defective intracellular-extracellular cationic balance which would have more serious consequences in muscle where it forms the triggering mechanism for excitation coupling. If such a membrane defect is indeed widely distributed in the tissues of dystrophic individuals, then it is likely that it should be sought in tissues other than muscle since the characteristic progressive degeneration of muscle in MD renders judgement about membrane function almost impossible.

Plate: 1

Figure: 1

A typical electrophoretogram of proteins on SDS-PAGE. The gel was stained with silver stain, track A: standard proteins- ovalbumin 43,000 daltons, carbonic anhydrase 29,000 daltons and cytochrome c 11,000 daltons; tracks B and C: 2,000 xg supernatant of mouse heart homogenate, 60 micrograms protein; D and E same as in B and C except 30 micrograms protein was loaded on the gel; F and G 2,000 xg supernatant of mouse liver homogenate, 25 micrograms protein; H and I same as in F and G except 50 micrograms protein was loaded on the gel.

A B C D E F G H I

Plate: 2

Figure: 2

Electrophoretograms of standard proteins on SDS-PAGE. Fig 2a) gel stained with coomassie blue stain, tracks A: bovine serum albumin 68,000 daltons; B: ovalbumin 43,000 daltons; C: carbonic anhydrase 29,000 daltons; D: mixture of standard proteins; E: trypsin inhibitor 21,000 daltons; F: myoglobin 17,000 daltons. Figure: 2b) gel stained with silver stain showing ovalbumin, carbonic anhydrase and cytochrome c. The arrow shows position of bovine serum albumin which did not show up in this gel. Figure: 2c) gel stained with silver stain, showing same set of standard proteins as in fig. 2b with additional phosphorylase b 90,000 daltons.

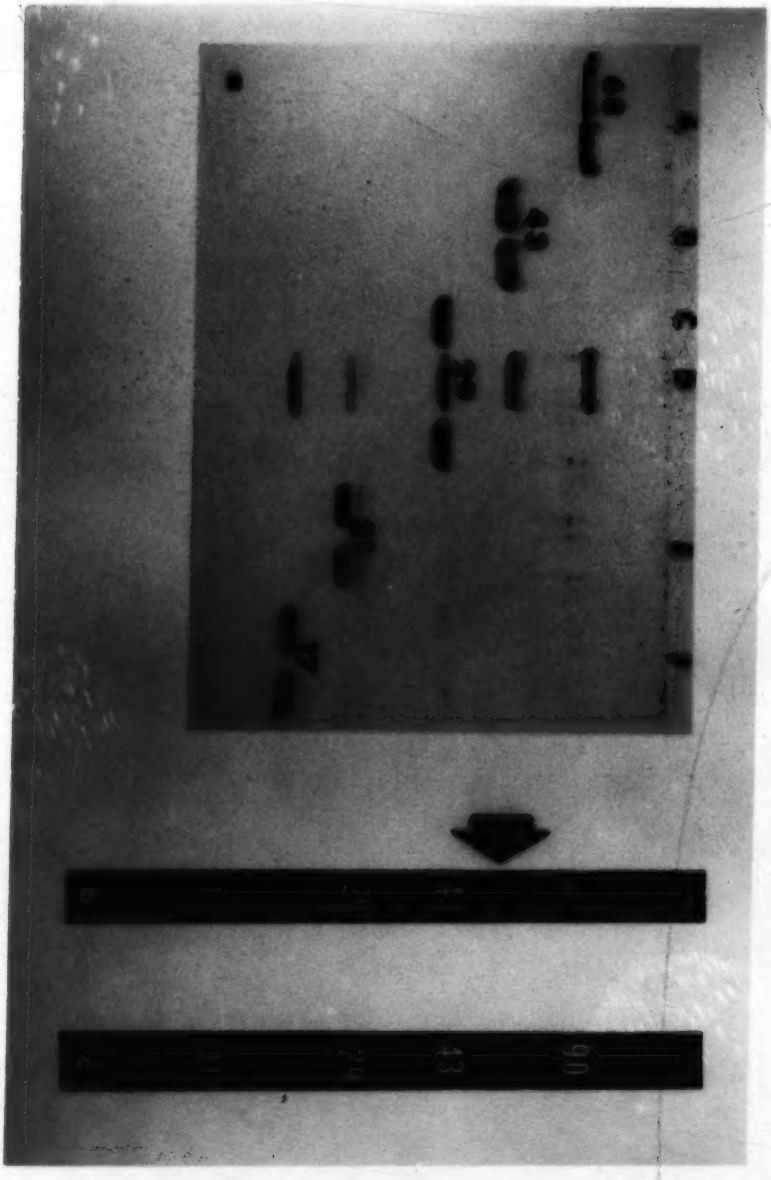
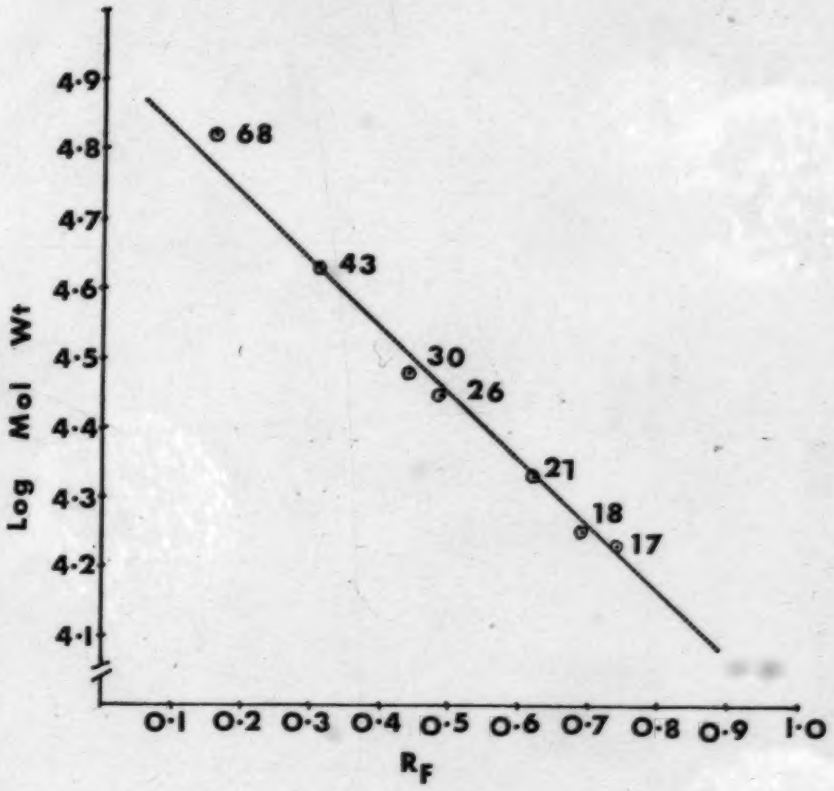


Figure: 3

Log molecular weight versus relative mobility (Rf) of seven standard proteins on SDS-PAGE. The numbers on the graph line shows mol. wt. X1,000 daltons. 68: bovine serum albumin; 43: ovalbumin; 26: chymotrypsinogen; 21: trypsin inhibitor; 18: lactoglobulin; 17: myoglobin. The data were obtained from an analysis of four gels.


Log Mol Wt
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
68
43
30
26
21
18
17
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
R_F

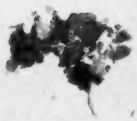


Plate: 3

Figure: 4

A typical electrophoretogram of erythtrocyte ghost membrane proteins obtained from normal hamster. Gel was stained with silver stain, track A: standard proteins, the number shows mol. wt. =x1,000 daltons; B: erythrocyte ghost membrane proteins showing a typical six band pattern the details of which are given under "RESULTS".

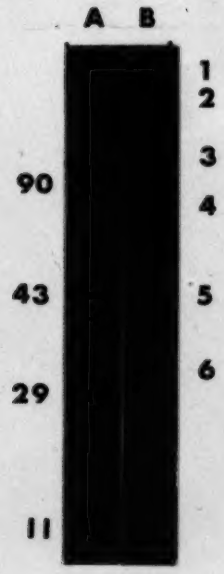

A
B
1
2
3
90
4
43
5
6
29
11

Plate: 4

Figure: 5

SDS-PAGE analysis of erythrocyte ghost membrane proteins from normal (track C) and dystrophic mice (track B). The arrow shows the difference in the peptide staining. Gel was stained with silver stain. Track A: standard proteins.

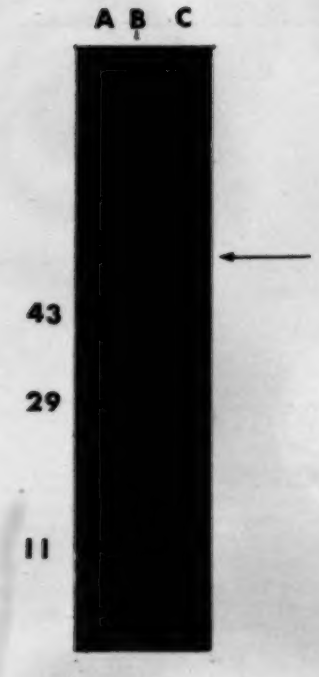

A B C
43
29
11

Figure: 6

Densitometric trace of SDS-PAGE electrophoretograms of eryhtrocyte ghost membrane proteins from a) normal and b) dystrophic mice. The arrow shows increased amount of 55,000 dalton band. Numbers in a) shows classification of erythrocyte membrane major proteins according to Fairbanks *et al*(1971).

a)
b)
6
5
4
3
2
1

Plate: 5

Figure: 7

Periodic acid Schiff stained electrophoretogram of erythrocyte ghost membrane from normal (tracks A and C) and dystrophic mice (B and D). The arrows show relative positions of three bands. Some bands did not show up in the photograph.

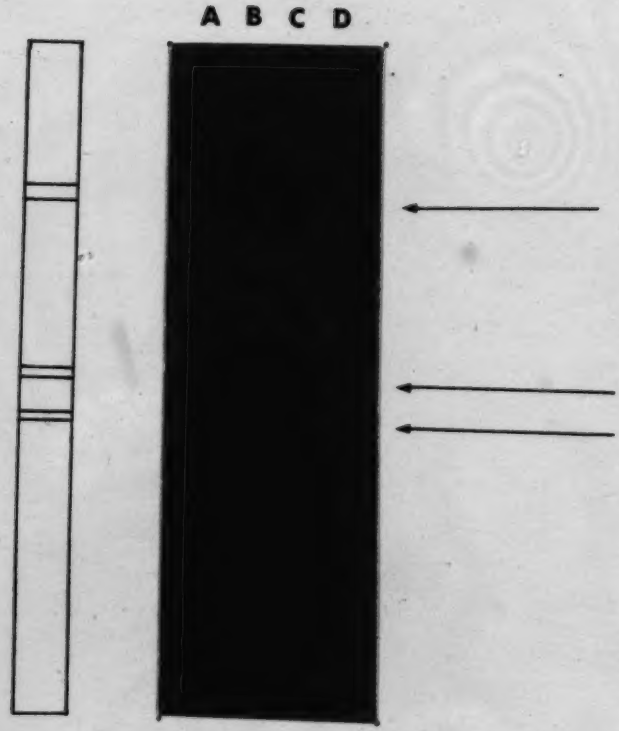

A B C D

Plate: 6

Figure: 8

SDS-PAGE analysis of erythrocyte ghost membrane proteins from normal (track C) and dystrophic hamsters (B and D). The arrow shows the difference in the peptide staining. Gel was stained with silver stain. Track A: standard proteins.

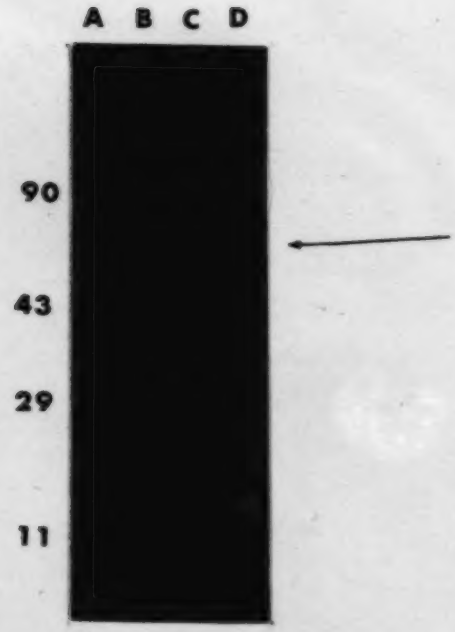

A B C D
90
43
29
11

Plate: 7

Figure: 9

Periodic acid Schiff stained electrophoretogram of erythrocyte ghost membrane from normal (tracks A and C) and dystrophic hamsters (B and D) The arrows show relative positions of four bands. Some bands did not show up in the photograph.

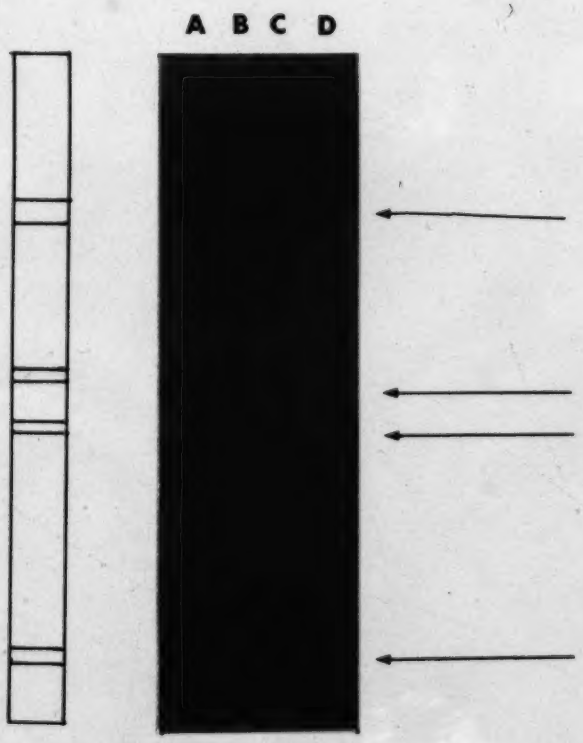

A B C D

Figure: 10

The pH gradient curve. The pH gradient was measured as described under "MATERIALS AND METHODS".

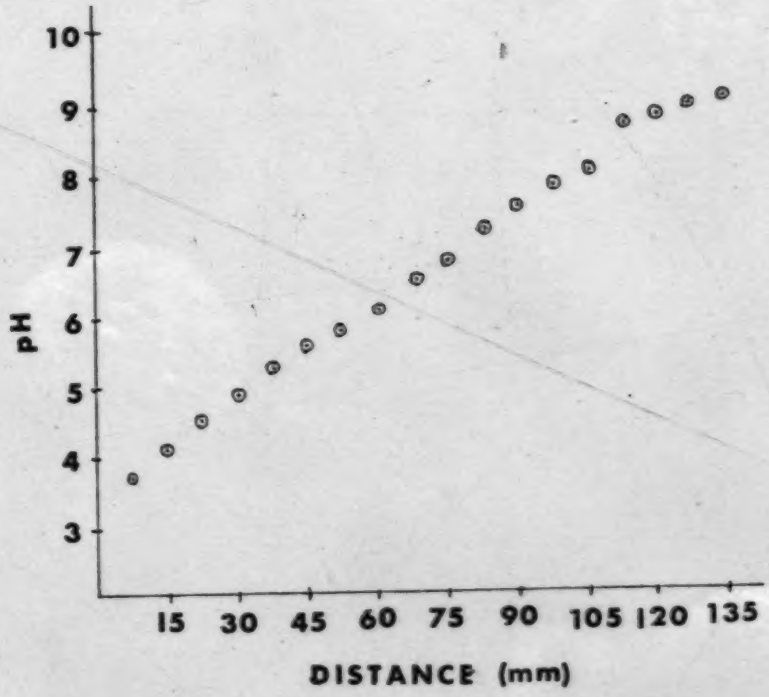

10
9
8
7
6
5
4
3
pH
15
30
45
60
75
90
105
120
135
DISTANCE (mm)

Plate: 8

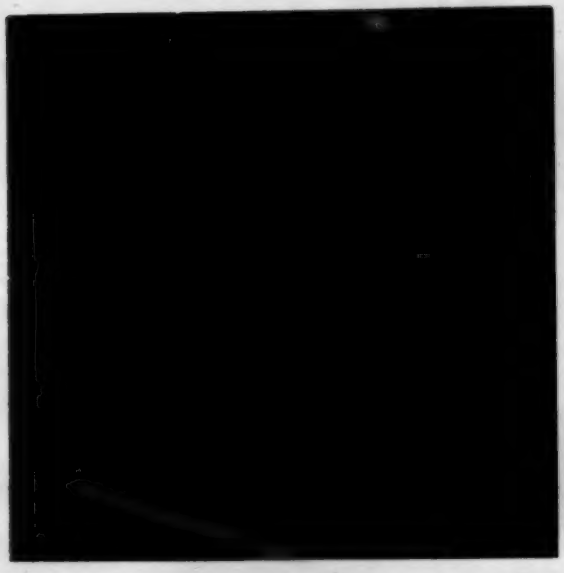
Figure: 11

A typical two-dimensional electrophoretogram of erythrocyte ghost membrane proteins from normal hamster; gel was stained with silver stain.

Plate: 9

Figure: 12

Two-dimensional gel analysis of eythrocyte ghost membrane proteins from a) normal and b) dystrophic mice. Circles in the fig b shows two spots which were stained much more intensely as compared to those in the fig a.



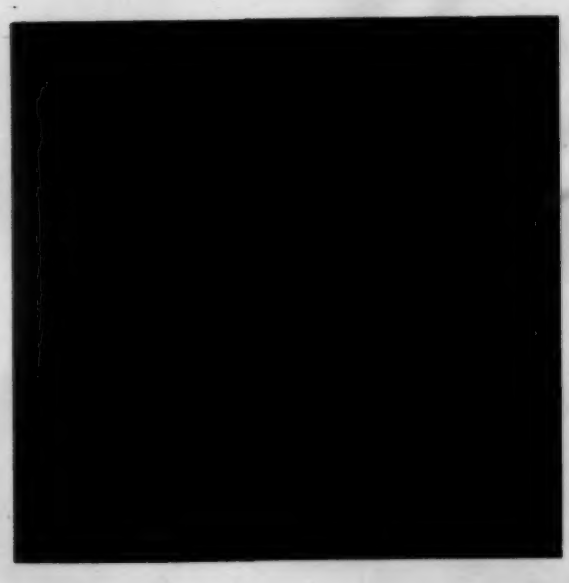


Plate: 10

Figure: 13

Two-dimensional gel analysis of eythrocyte ghost membrane proteins from a) normal and b) dystrophic hamsters. The arrow in fig. b shows the additional spot from dystrophic prepartions.

Plate: 11

Figure: 14

Scanning electron micrographs of erythrocytes showing a normal biconcave discocyte (fig. a) and a triconcave knizocyte (fig. b) in the right half of the photographs. The cells were obtained from normal hamsters. The right half of the photograph is 4.1 times the left half.

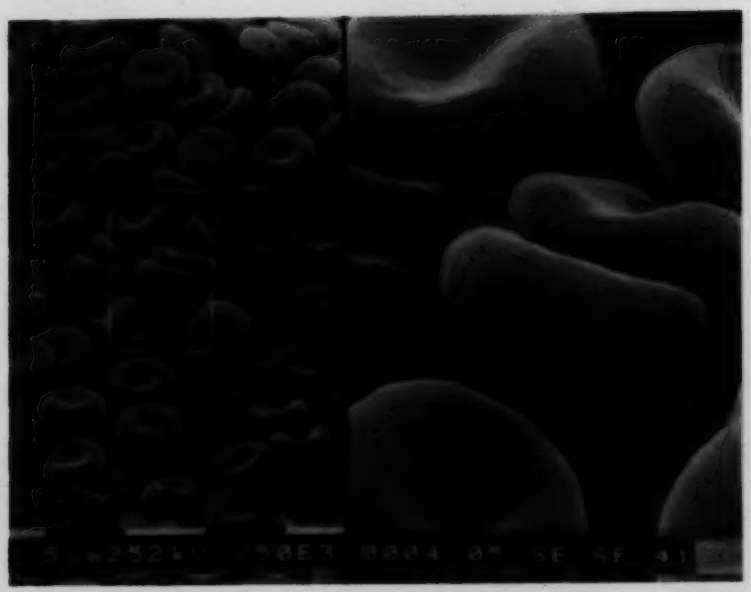


Plate: 12

Figure: 15

Scanning electron micrographs of erythrocytes showing a cup shaped stomatocyte (fig. c) and a star shaped echinocyte (fig. d) in the right half of the photographs. The cells were obtained from dystrophic and normal mice respectively. In fig. c the right half is 4.1 times the left half while in fig. d it is 2.2 times.

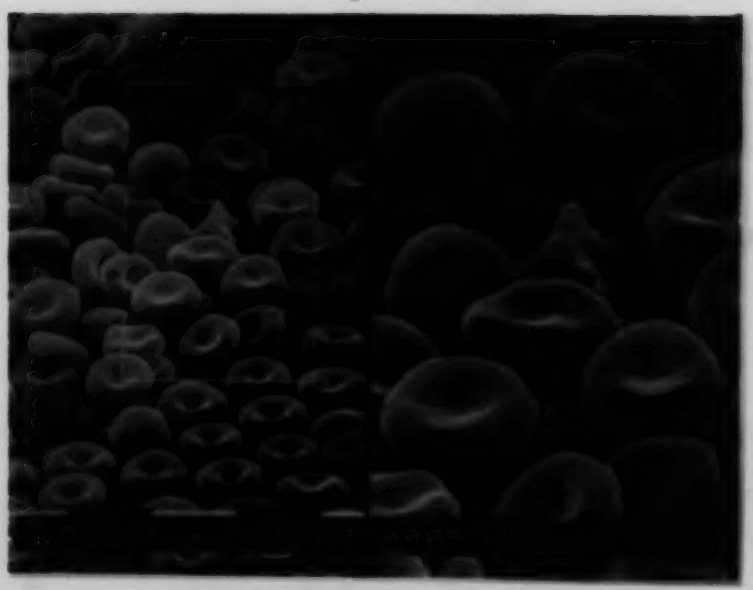


Plate: 13

Figure: 16

Scanning electron micrographs of erythrocytes obtained from normal (fig. a) and dystrophic (fig. b) mice. The arrows in fig. b show stomatocytes.

a

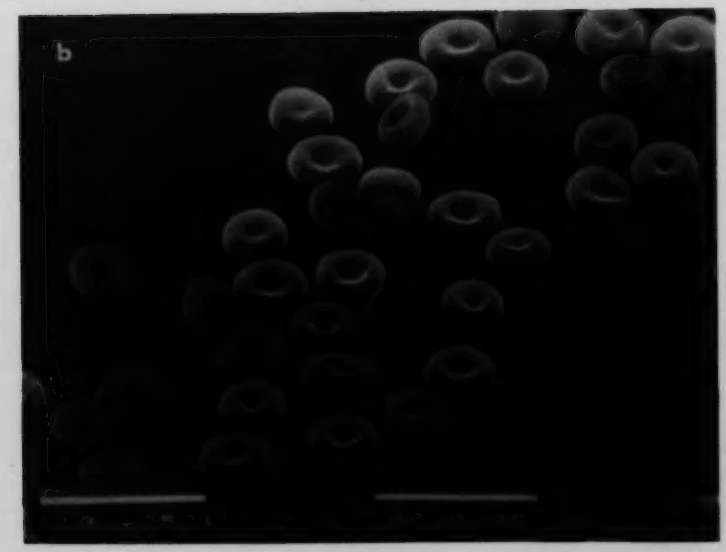

b

Table 1: Percentage of distorted erythrocytes in Normal and Dystrophic Mice.

Controls

Animal no.	Total no. of cells counted	Biconcave cells	Distorted cells	%distorted cells
1	165	147	18	11
2	254	236	18	7
3	73	69	4	5
4	248	228	20	8
Total	**740**	**680**	**60**	**8**

Dystrophic

Animal no.	Total no. of cells counted	Biconcave cells	Distorted cells	% distorted cells
1	383	149	234	61
2	62	40	22	35
3	156	51	105	67
4	292	125	167	57
Total	**893**	**365**	**528**	**59**

Plate: 14

Figure: 17

Scanning electron micrographs of erythrocytes obtained from normal (fig. a) and dystrophic (fig. b) hamsters. The arrows in fig. b show echinocytes.

Table 2: Percentage of distorted erythrocytes in Normal and Dystrophic Hamsters.

Controls

Animal no.	Total no. of cells counted	Biconcave cell	Distorted cells	% distorted cells
1	150	135	15	10
2	169	151	18	10
3	83	75	8	10
4	101	93	8	8
Total	**503**	**454**	**49**	**10**

Dystrophic

Animal no.	Total no. of cells counted	Biconcave cells	Distorted cells	% distorted cells
1	147	50	97	66
2	155	79	76	49
3	90	49	41	46
4	125	34	91	73
Total	**517**	**212**	**305**	**59**

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APPENDICES

APPENDIX I

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

In systems such free eletrophoresis and ion exchange chromatography, migration of protein is mainly dependent on the molecular net charge. However, when they move in an electric field through a solid medium such as polyacrylamide gel, their movment is affected very strongly by their size (Ornstein, 1964; Davis, 1964). Thus, if different proteins have equal charge, their migration becomes the function of their size. Sodium dodecyl sulfate (SDS) is an ampiphile which binds to protein in a nonspecific manner (Reynolds and Tanford, 1970a). It has been shown that a wide variety of proteins bind identical amount of SDS on a gram to gram basis when saturated (Reynolds and Tanford, 1970b). The protein then has an overall negative charge that masks its intrinsic charge (Weber and Osborn, 1969) resulting in a constant charge to mass ratio for proteins (Reynolds and Tanford, 1970a; 1970b). SDS binding also induces a conformational change in the polypeptide chains leading to a uniform rod-like configuration, the length of the structure being proportional to its polypeptide chain length and thus its molecular weight (Reynolds and Tanford, 1970b). Thus, in the presence of SDS, migration of a polypeptide on polyacrylamide gel becomes directly dependent on its molecular weight.

Theoretical treatment for obtaining mol. wt. values from relative mobility values of protein-SDS complexes in gels have been given by Neville (1971). Acrylamide gels are considered to have an average pore size (Chrabach and Rodbard, 1971) which is inversely related to the acrylamide concentration, T. When macromolecules are forced through the gel by the electric field, the relative mobility of the macromolecule in the gel, Rf, is retarded by the gel compared to the relative free mobility in buffer, Yo. These relationships, first noted by Ferguson (1964), define the

retardation coefficient, Kr

$$-Kr = \frac{\log Rf - \log Yo}{T}$$

Within certain ranges Kr is a uniform function of the macromolecular hydrodynamic radius or the Stokes radius, Rs, which in turn is a uniform function of protein mass, M, for protein-SDS complex. Therefore, Kr is a uniform function of M. Because Protein-SDS complexes behave, in electrophoresis, as free draining coiles and have approximately constant values of Yo (Reynolds and Tanford, 1970b) irrespctive of M, in a single gel of fixed T, Rf is uniform function of M (within the specified range).

APPENDIX II.

Isoelectric Focusing

Two-dimensional electrophoresis (2-DE) involves separation of proteins according to two different properties. In the present investigation, IEF has been used in the first dimension and SDS-PAGE in the second dimension. Theoretical basis of protein separation in SDS-PAGE has been discussed in the APPENDIX I therefore, only the phenomenon of separation according to charge will be discussed. On IEF gels proteins are separated depending on the charge, as reflected in the isoelectric point (Anderson and Anderson, 1978). The net charge of a protein molecule in an acidic solution is positive because most amino group carry a positive net charge and most carboxylic groups are protonated and therefore electrically uncharged. If the pH is gradually increased, the number of carboxylic groups which carry a negative charge will increase and the number of positively charged groups will decrease. Thus, at a certain pH value, the isoionic point, the net charge of the protein molecule will become zero.

In IEF, a stable pH gradient is established in gel by using low mol. wt. carrier ampholytes. If a protein is put into this system at a pH other than its isoionic point, it will carry a net charge and so it will migrate to an appropriate electrode under the influence of electric field. As it passes through the pH gradient, the net charge of the molecule will change. The protein will eventually reach a pH, where its net charge will be zero and it will stop migrating. This is the isoelectric point (pI) of the protein. The pH at this point is, thus, equal to the isoelectric point which is very close to, or equal to, the isoionic point (Vesterberg 1971) The consequence of this is that every protein will migrate to and focus at its respective isoelectric point in a stable pH gradient irrespective of its origin in the gel at the time the current was applied.

The pH gradient is generated by isoelectric focusing of special buffer substances, which are called "carrier ampholytes". These are

generally isomers and homologs of aliphatic polyamino polycaboxylic acids. The ampholytes are commercially available as mixtures of several specific pH ranges, such as, 3-10, 6-8. This means that most of the ampholytes in a given sample have their pI in the corresponding range and are thus able to yield a pH gradient covering that range.

Resolving power of IEF (the smallest pI difference, ΔpI, of proteins which still permits their separability) can be calculated by using the following equation

$$\Delta pI = K \sqrt{\frac{D(dpH/dx)}{-E(du/dpH)}}$$

where, K is a factor defined by the criterion for separation (numerical value of 3 has been recommended); D=diffusion coefficient in cm per sec. of the protein; x=coordinate along the direction of the current; E=the field strength at the point of focusing in volts per cm; u=electric mobility at pH close to the pI of the protein, in cm per volt per sec. The derivative dpH/dx is the value of the pH gradient. Thus, a narrow pH range will yield a high resolving power.

APPENDIX III

Scanning Electron Microscopy

The principal advantage of the SEM, as compared to the conventional transmission electron microscope, generally lies in a somewhat broader area of information transfer rather than in resolution or greater available detail of the image (Hayes and Pease, 1968). The SEM might be described as consisting of two systems: the probing system into which the specimen is placed and the display system which forms the visual image. In the probing system, an electron beam is focused on the surface of specimen at a point. As the electrons interact with the material of the specimen, they induce a variety of radiations such as, characteristic X-rays, secondary electrons (emitted from just below the specimen surface) and reflected primary electrons. These electrons (mainly the secondary electrons) are collected by a detector, the signal amplified and is used to form an "image" of that point of the specimen surface on the display cathode tube. The brightness of the image depends on the amount of radiation (secondary electrons) leaving a point on the specimen.

The probing beam is then moved to an adjacent spot on the specimen and the information, from that spot, is transfered to the adjoining point on the display cathode ray tube. This is repeated until all points of the specimen have been covered. In practice, the points move with great speed so that, to the eye, a complete image is formed. To obtain a permanent record these spots can be collected over a long period of time on photographic film producing an integrated picture of the specimen.

In SEM, magnification is defined as the ratio of the linear size of the display raster (array of lines) to the size of the specimen raster. The useful range of magnification is dictated by the resolving power of the instrument (upper limit) and by the lens design and placement of the specimen (lower limit) and is of the order of ten times at the low end to

fifty thousand times at the upper end.

Resolution in SEM is limited ultimately by the diameter of the probe spot. To obtain maximum current density, in terms of a minimum diameter of the probing beam of electrons, in a system free of abberations, the minimum diameter of spot can be calculated by the following equation given by Langmuir (1937)

$$do^2 = \left(\frac{4i}{0.6\ 7T\ Jc\ \alpha^2} \right) \frac{kT}{eV}$$

where, do is the Gaussian probe diameter, Jc is the emission current density of the cathode, eV is the electron energy, iis the probe current, α is the semi-angle of convergence of the electron probe, k is Boltzmann's constant and T is the absolute temperature of the cathode.

In addition to this minimum diameter imposed by the basic electron optical consideration, there are also other factors such as spherical aberrations, chromatic aberrations and astigmatism of lens which limit the resolving power. Currently available scanning electron microscopes generally offer a resolution of 50 angstroms.